Original Article

Islet Surface Heparinization Prevents the Instant Blood-Mediated Inflammatory Reaction in Islet Transplantation

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OBJECTIVE—In clinical islet transplantation, the instant blood-mediated inflammatory reaction (IBMIR) is a major factor contributing to the poor initial engraftment of the islets. This reaction is triggered by tissue factor and monocyte chemoattractant protein (MCP)-1, expressed by the transplanted pancreatic islets when the islets come in contact with blood in the portal vein. All currently identified systemic inhibitors of the IBMIR are associated with a significantly increased risk of bleeding or other side effects. To avoid systemic treatment, the aim of the present study was to render the islet graft blood biocompatible by applying a continuous heparin coating to the islet surface.

RESEARCH DESIGN AND METHODS—A biotin/avidin technique was used to conjugate preformed heparin complexes to the surface of pancreatic islets. This endothelial-like coating was achieved by conjugating barely 40 IU heparin per full-size clinical islet transplant.

RESULTS—Both in an in vitro loop model and in an allogeneic porcine model of clinical islet transplantation, this heparin coating provided protection against the IBMIR. Culturing heparinized islets for 24 h did not affect insulin release after glucose challenge, and heparin-coated islets cured diabetic mice in a manner similar to untreated islets.

CONCLUSIONS—This novel pretreatment procedure prevents intraportal thrombosis and efficiently inhibits the IBMIR without increasing the bleeding risk and, unlike other pretreatment procedures (e.g., gene therapy), without inducing acute or chronic toxicity in the islets. ***Diabetes*** **56:2008–2015, 2007**

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APC, activated protein C, IBMIR, instant blood-mediated inflammatory reaction; MCP, monocyte chemoattractant protein; TAT, thrombin antithrombin.

Clinical islet transplantation is a promising therapy for restoring β-cell function in patients with type 1 diabetes, but poor engraftment of the transplanted islets limits the application of the procedure. Despite the use of islets from multiple donors, the total islet mass in insulin-independent patients is estimated to be only 30% that of a nondiabetic individual (1,2), suggesting that a vast majority of the graft is lost in the early posttransplantation period.

We and others have demonstrated that innate immune reactions may constitute one of the key factors involved in the destruction of the islet graft (3,4). Tissue factor, monocyte chemoattractant protein (MCP)-1, and other inflammatory mediators trigger an injurious thrombotic/inflammatory reaction, engaging the coagulation and complement systems, platelets, and polymorphonuclear neutrophils, when islets come in contact with blood in the portal vein (4–8). We have termed this response the instant blood-mediated inflammatory reaction (IBMIR). As a result of a tissue factor–elicited activation of coagulation, thrombin antithrombin (TAT) levels are consistently increased immediately after intraportal infusion of the islet graft, suggesting a regular occurrence of subclinical intraportal thrombosis in clinical islet transplantation. A simultaneous nonphysiological rise in C-peptide levels indicates islet damage and loss of transplanted tissue (3,4). Consistent with this notion is the observation that increased magnitude of the IBMIR (expressed as TAT levels) is correlated with poor clinical outcome (3). Thus, attenuation of IBMIR has been reckoned of prime importance for success in clinical islet transplantation (9).

A number of drugs have been identified that inhibit IBMIR, including the thrombin inhibitor Melagtran (10), the tissue factor inhibitor active site-inactivated FVIIa (active site inactivated seven) (4), activated protein C (APC) (11), and dextran sulfate (12). The clinical application of these drugs is limited by the increased risk of bleeding, especially taking into consideration the fact that the portal vein is accessed via the transhepatic route in most patients.

We have previously shown that soluble heparin, which is used in clinical islet transplantation, has no significant effect on the IBMIR (13). However, immobilization of the heparin on artificial surfaces, which mimics the protective feature of the endothelial cell lining on the native vascular wall, is associated with high compatibility: i.e., inhibition of coagulation and complement activation and a reduction

in cell and platelet adhesion and activation (14). Heparin coatings have previously been successfully applied to biomaterials such as extracorporeal membrane oxygenators and stents (14). Here, we present a novel large-scale technique for applying a coherent heparin coating to isolated islets in vitro, before transplantation, as a means of effectively abrogating the IBMIR. Unlike some virus-mediated transduction strategies, this technique does not affect the functional capacity of the islets, and it circumvents the side effects associated with gene therapy and the bleeding risk of systemic IBMIR inhibitors.

RESEARCH DESIGN AND METHODS

Protocols for the isolation of human islets from cadaver donors and for all animal experiments were approved by the regional research ethics committees of Uppsala University, and experiments were performed in accordance with local institutional and Swedish national rules and regulations.

Islet isolation and culture. Human pancreata were obtained from normoglycemic cadaveric donors after appropriate consent for multiorgan donation. Islets of Langerhans were isolated using a modification of the previously described semiautomated digestion-filtration method (15), followed by purification on a continuous density gradient in a refrigerated cell processor (COBE 2991; COBE Blood Component Technology, Lakewood, CO).

The islet preparations were of good quality and were designated for experimental use because the total islet volume was too low for use in clinical transplantation. Islet purity was determined by staining with diphenylthiocarbazone. Islet preparations were 70–90% pure.

After the isolation and purification, the islet preparations were placed in untreated single transfer packs for platelets 1300, the maximum volume of the bag (Baxter Medical, Sweden), and kept at 37°C in an atmosphere of 5% CO_2 in humidified air in CMRL 1066 culture medium (Mediatech, Herndon, VA) supplemented with 10 nmol/l nicotinamide (Sigma Aldrich, Schnelldorf, Germany), 10 mmol/l HEPES buffer (Invitrogen, Paisley, Scotland), 0.25 μg/ml fungizone (Invitrogen), 50 μg/ml gentamicin (Invitrogen), 2 mmol/l L-glutamine (Invitrogen), 10 μg/ml Ciprofloxacin (Bayer, Leverkusen, Germany), and 10% (vol/vol) heat-inactivated human serum.

Porcine islets were isolated and purified at the Third Medical Department, Justus-Liebig-University, Giessen, according to published protocols (16). Isolation of mouse islets was performed using a collagenase digestion method as previously described (17).

Cell surface heparinization. Human, porcine, and mouse islets were biotinylated by incubating the islets for 1 h at 37°C in culture medium containing SNL biotin (EZ-Link Sulfo-NHS-LC-Biotin), tissue factor-P (EZ-Link tetraphenyl ester), or tissue factor-P biotin (EZ-Link tetrephenyl ester-PEO-Biotin) (Pierce Biotechnology, Rockford, IL) at 1 mg/ml. The islets were then rinsed by changing the culture medium three times. In the next step, the islets were incubated for 30 min at 37°C in culture medium supplemented with 1 mg/ml avidin (Pierce Biotechnology) and again rinsed by changing the culture medium three times. Finally, 1 mg/ml macromolecular conjugates of heparin, ~70 heparin molecules covalently linked to an inert carrier chain (Corline Systems, Uppsala, Sweden), in culture medium were allowed to bind to the biotin/avidin coating for 60 min at 37°C. The islets were finally rinsed by changing the culture medium three times.

Although SNL biotin and tissue factor-P biotin gave similar results, a direct comparison showed slightly better results with SNL biotin, and, hence, this substance was chosen for experiments in the allogeneic porcine and syngeneic mice islet transplantation model.

The endothelial cell surfaces of human pancreatic arteries were modified by the same method (using SNL biotin). Each artery segment was cut in half; one-half was used for heparinization and one-half as a control. The artery segments, 3–4 cm in length, were connected at both ends to 20-cm–long pieces of polyethylene tubing (4 mm inner diameter). Ringer's acetate buffer (5 ml) was transferred into the segments and rocked back and forth over the endothelial lining several times for 1 min. The artery segments were then coated as described above, using the same regents, washes, and incubation times as described for the islets. The control artery was filled with 5 ml Ringer's solution and submerged in a Petri dish in the same solution during the heparinization process. Immediately after the immobilization process, both samples were snap frozen in liquid nitrogen and stored for later analysis.

Confocal microscopy. The degree of heparinization of the islets and of cryosections of heparinized pancreatic arteries was visualized by confocal microscopy after incubating the tissues at room temperature for 15 min with antithrombin (1 unit/ml) labeled with Alexa Fluor 488. Propidium iodide (Sigma, St. Louis, MO) was used to stain nonviable cells, and Hoechst 33342 (Sigma) was used to detect cell nuclei in heparinized islets. Images were acquired with a confocal microscope (Zeiss LSM 510 Meta; Carl Zeiss, Jena, Gemany) equipped with an Axiovert 200 microscope stand. Z-stacks of the islet surfaces were acquired using the 488-nm laser line, a 20× objective, and a 505–550 bit plane filter. Three-dimensional projections of the acquired z-stacks were analyzed using Imaris software (Bitplane, Zurich, Switzerland). Images were acquired at 20× magnification. Because of the high level of autofluorescence emission within the spectrum of Alexa Fluor 488, autofluorescence was determined in lambda mode, and images were then acquired in fingerprinting mode using the appropriate spectra.

Viability tests

Dynamic perfusion system. Insulin secretion in response to glucose stimulation in a dynamic perfusion system was assessed on day 1 ($n = 3$). Islets were initially perfused with 1.67 mmol/l glucose, then with 16.7 mmol/l, and again with 1.67 mmol/l. During the 120-min perfusion, fractions were collected at 6-min intervals. The concentration of insulin was analyzed using a commercial enzyme-linked immunosorbent assay kit (Mercodia, Uppsala, Sweden). Values are given as picomoles per liter.

Syngeneic islet transplantation in alloxan diabetic mice. All mice (25–30 g) were made diabetic by intravenous administration of alloxan (75 mg/kg body wt) as elsewhere described (18). Diabetes was confirmed by the presence of hyperglycemia (>25 mmol/l blood glucose for 2 consecutive days). Four to six days after the induction of diabetes, either 300 heparinized (using SNL biotin; $n = 7$) or 300 untreated control ($n = 7$) islets (in this model, 300 islets is the islet mass required for reversal of diabetes in mice receiving control islets) were transplanted under the left kidney capsule into B6 mice (Bomholdtgaard, Ry, Denmark) on a C57BL/6J background, as previously described (19). After transplantation, blood glucose was measured every third day for 4 weeks. At 5 weeks after the transplantation, all mice underwent an intraperitoneal glucose tolerance test by injection of 250 μl of a 30% glucose solution. Blood glucose concentrations were determined before the glucose administration (time point 0) and 15, 30, 60, and 120 min after the injection. At the end of the experiment, to ensure persistent absence of endogenous pancreatic insulin production, the graft-bearing kidney was surgically removed from all animals. Reversal of diabetes was defined as blood glucose >25 mmol/l in two consecutive measurements. After the nephrectomy, all grafts were retrieved, paraffin embedded, and evaluated by immunohistochemical staining for morphology and the presence of insulin.

In vitro tube-loop model. The capacity of surface heparinized and control islets to induce IBMIR was evaluated using a modification of an in vitro loop model as previously described (4,13): 2 μl either heparinized (using tissue factor P or SNL biotin) or untreated control islets were resuspended in 100 μl CMRL 1066. Fresh ABO-compatible human blood (7 ml) was added to each loop before perfusion of the islets. In similar experiments, blood loops with heparinized islets (using SNL biotin) were compared with untreated islets, with and without the addition of the specific thrombin inhibitor Melagatran (4 μmol/l), which has previously been shown to be an efficient inhibitor of the IBMIR (10). In both experiments, control loops with 100 μl CMRL 1006 alone were included. To generate blood flow, the loops were placed on a rocking device inside a 37°C incubator for 60 min. To monitor the IBMIR, blood samples of 1 ml were collected in EDTA (4.2 nmol/l, final concentration) before perfusion and at 5, 15, 30, and 60 min after the start of the perfusion.

Platelet counts were assessed using a Coulter AcT diff analyzer (Beckman Coulter, Miami, FL). Plasma concentrations of TAT complexes were quantified using a commercially available enzyme-linked immunosorbent assay kit (Enzygnost TAT; Dade Behring, Marburg, Germany). The complement activation product C3a was quantified as previously described (20).

Intraportal transplantation of adult porcine islets. Five pairs of piglets of Swedish mixed-country breed, weighing 11–16 kg, were anesthetized using xylazine (2.2 mg/kg), tiletamin/zolazepam (6 mg/kg), and atropine (0.04 mg/kg) as a premedication given intramuscularly in the neck and then were given fetanyl (15–20 μg/kg) at the onset of anesthesia. They were orally intubated using a cuffed endotracheal tube and ventilated with mechanical ventilation in volume-controlled mode. A continuous infusion of ketamine (30 $mg \cdot kg^{-1} \cdot h^{-1}$), fetanyl (5 $\mu g \cdot kg^{-1} \cdot h^{-1}$), and midazolam (0.5 $mg \cdot kg^{-1} \cdot h^{-1}$) was given. Pancuronium (0.3 mg/kg) was given intravenously. No systemic anticoagulant was administrated. A catheter was placed in the superior mesenteric vein. Heparinized (using SNL biotin) or untreated control adult porcine islets (7,500 islet equilvalent/kg body wt) isolated from the same donor animal were infused intraportally, using a bag system, over 5 min into each pair of pigs. Blood samples were collected into EDTA tubes before and 15, 30, and 60 min after islet transplantation. After 60 min, the pigs were killed with an overdose of KCl, and the livers were excised and the portal system exposed. Blood clots and liver biopsies were taken and fixed in paraformaldehyde or snap frozen in liquid nitrogen.

Immunohistochemical staining. Transplanted grafts, liver biopsies, and clots retrieved from portal vessels were fixed in paraformaldehyde (Histofix;

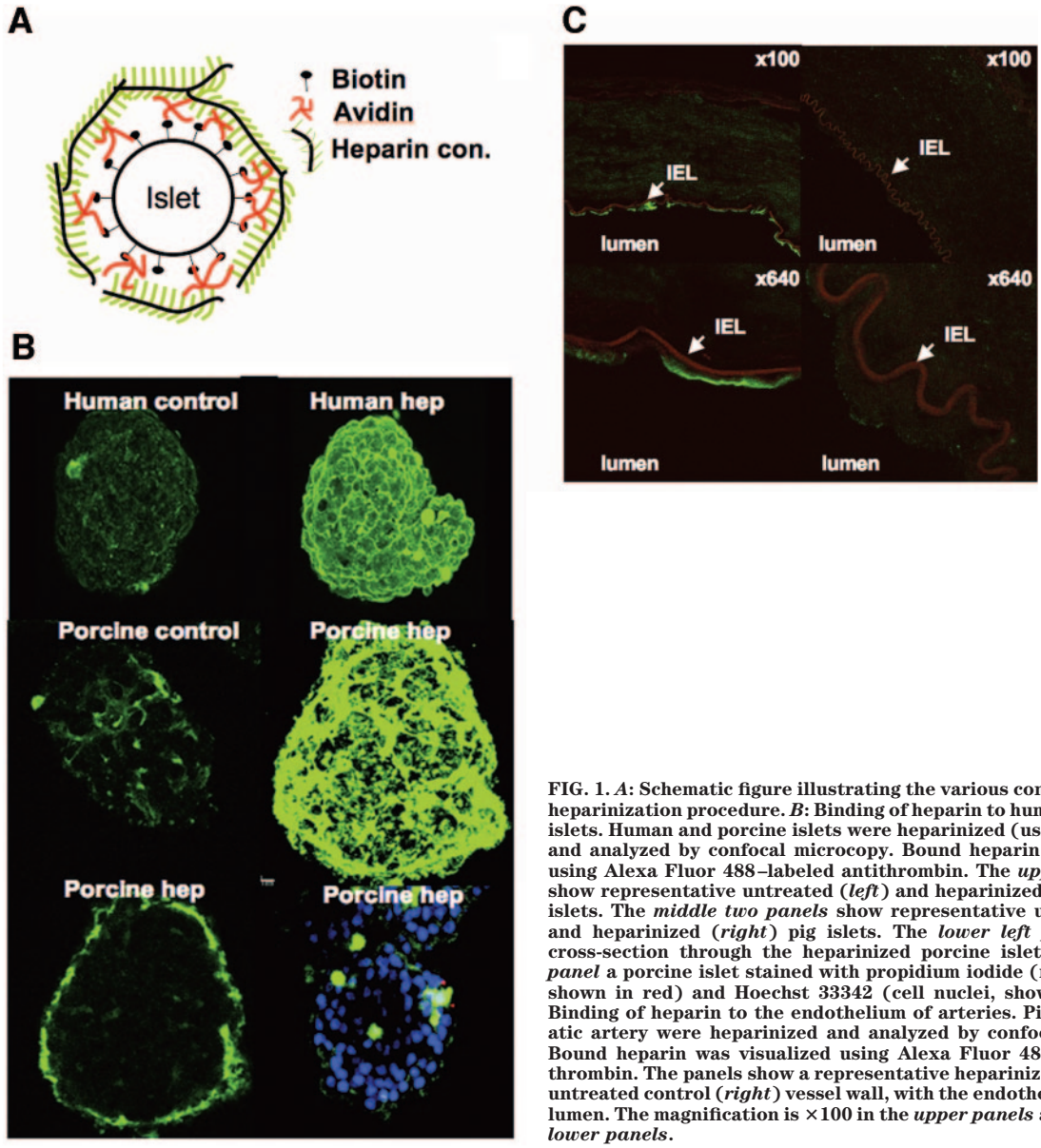

FIG. 1. ***A*****: Schematic figure illustrating the various components of the heparinization procedure.** ***B*****: Binding of heparin to human and porcine islets. Human and porcine islets were heparinized (using SNL biotin) and analyzed by confocal microcopy. Bound heparin was visualized using Alexa Fluor 488–labeled antithrombin. The** ***upper two panels*** **show representative untreated (*left*) and heparinized (*right*) human islets. The** ***middle two panels*** **show representative untreated (*left*) and heparinized (*right*) pig islets. The** ***lower left panel*** **shows a cross-section through the heparinized porcine islet and the** ***right panel*** **a porcine islet stained with propidium iodide (nonviable cells, shown in red) and Hoechst 33342 (cell nuclei, shown in blue).** ***C*****: Binding of heparin to the endothelium of arteries. Pieces of pancreatic artery were heparinized and analyzed by confocal microscopy. Bound heparin was visualized using Alexa Fluor 488–labeled antithrombin. The panels show a representative heparinized (*left*) and an untreated control (*right*) vessel wall, with the endothelium facing the lumen. The magnification is ×100 in the** ***upper panels*** **and ×640 in the** ***lower panels*****.**

Histolab Products, Västra Frölunda, Sweden), embedded in paraffin, sectioned on a Leica Jung RM 2055 microtome at 4 μm, and air-dried at 37°C overnight. The sections were kept at room temperature until dewaxing. They were then fixed and stained for insulin using Rabbit Envision (DAKO, Copenhagen, Denmark).

Statistics. All results are presented as means ± SEM. Prism, version 4.0a, for Macintosh was used for statistical calculations. The in vitro loop studies were evaluated using Friedman's ANOVA. Significance was determined at $P < 0.05$. For evaluation of the in vivo allogeneic porcine islet transplantation experiments, the Mann-Whitney nonparametric test (double tailed) was used.

RESULTS

Islet surface heparinization. Confocal microscopy of the heparinized islets consistently revealed evenly distributed fluorescence, demonstrating that the heparin coat covered the complete islet surface (Fig. 1*B*). Cross-sections of the islets showed that the heparin bound strictly to the surface of the islets, as reflected by the binding of Alexa Fluor 488–labeled antithrombin. This analysis confirmed that the heparinized islets were surrounded by a coherent and smooth coating of heparin, which was strictly confined to the surface of the islet. The coating was present on cultured human islets for at least 72 h but was not detectable on transplanted mouse islets after 4–5 weeks under the kidney capsule.

Islets treated with heparin conjugates alone, with heparin conjugates after biotinylation (excluding the avidin step) or with heparin conjugates after treatment of the islets with avidin (excluding the biotin step), demonstrated no binding of Alexa Fluor–labeled antithrombin (data not shown).

Heparinization of endothelial cell surfaces. Sections of heparinized vessel walls treated with Alexa Fluor 488–labeled antithrombin demonstrated a distinct fluorescence following the endothelial cell lining, with sparse distribution into the subendothelium (Fig. 1*C*, *left panel*). No fluorescence was detected below the external elastic membrane or in the muscularis. Untreated control vessel wall showed no binding of Alexa Fluor 488–labeled antithrombin (Fig. 1*C*, *right panel*).

Islet viability. The macroscopic appearance of the islets after heparinization did not differ from that of untreated control islets, and there was no sign of islet loss, even after several days of culture. However, the heparinized islets

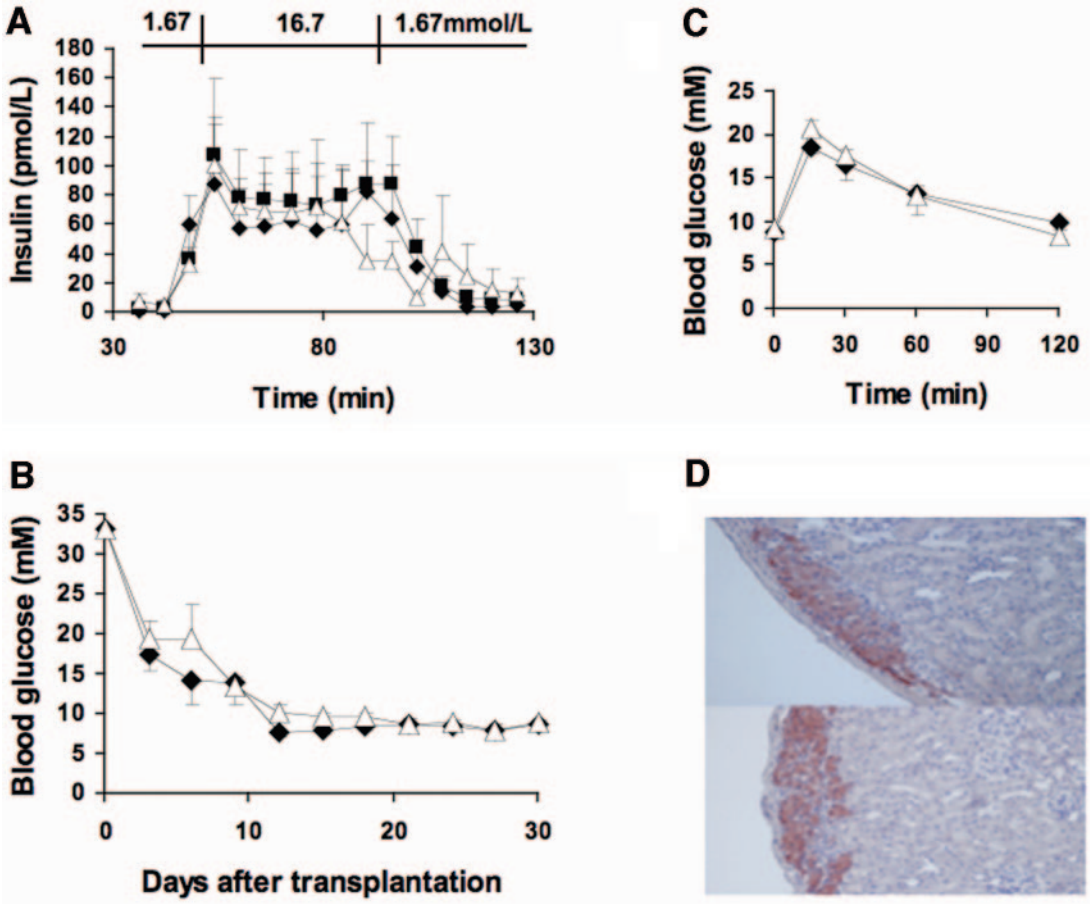

FIG. 2. *A:* **Insulin release in response to a glucose challenge. Insulin release from heparinized (■, tissue factor P biotin; △, SNL biotin) and control (◆) islets that had been cultured for 24 h (n = 3). The islets were sequentially stimulated with 1.67, 16.7, and 1.67 mmol/l glucose. The mean ± SEM is shown.** *B*: **Blood glucose levels after syngeneic islet transplantation into diabetic mice. Blood glucose levels of alloxan-induced diabetic wild-type B6 mice transplanted with heparinized (△; n = 7) or untreated control (◆; n = 7) islets. The mean ± SEM is shown.** *C*: **Intraperitoneal glucose tolerance test. Reversal of hyperglycemia following an intraperitoneal glucose injection into alloxan-induced diabetic wild-type B6 mice carrying heparinized (△; n = 7) or untreated control (◆; n = 7) islets. The mean ± SEM is shown.** *D*: **Immunohistochemical evaluation. Alloxan-induced diabetic wild-type B6 mice were transplanted with heparinized or untreated islets. The transplanted tissue was retrieved after 5 weeks and analyzed by immunohistochemical staining. The figure shows the presence of insulin in grafts 5 weeks after transplantation in animals that had received untreated (*upper panel*) or heparinized (*lower panel*) islets (×20 magnification).**

preserved their integrity over time, with no tendency to adhere to plastic surfaces or to other islets.

Human and porcine islets were stained with propidium iodide and Hoechst 33342 and then analyzed by confocal microscopy. These analyses indicated that >90% of the cells were viable, with no difference seen between heparinized and control islets (Fig. 1*B*). Heparinized and untreated control islets from three islet preparations responded similarly to glucose stimulation, demonstrating that the normal biphasic insulin secretion was not affected by the heparinization procedure (Fig. 2*A*).

In a syngeneic transplantation model, all mice that received either heparinized (n = 7) or untreated control (n = 7) islets had normalized blood glucose levels within 10 days after transplantation and subsequently responded equally well to an intraperitoneal glucose tolerance test (Fig. 2*B* and *C*, respectively). After removal of the graft-bearing kidney, all 14 mice immediately became hyperglycemic and remained so for the 4 days until they were killed, demonstrating that regeneration of islets in the mouse pancreas had not occurred. Positive insulin staining and intact morphology indicated successful engraftment and survival of both heparin and untreated control islets (Fig. 2*D*).

Long-term graft survival was tested by transplanting heparinized (using SNL biotin) and untreated human islets under the kidney capsule in B6 athymic nude (*nu/nu*) mice. After 4 weeks, the grafts were retrieved and paraffin sections evaluated for morphology and for the presence of insulin by immunohistochemical staining. The results demonstrated successful engraftment and survival of both heparin and untreated control islets (data not shown).

Perfusion of human islets with fresh human ABO-identical blood in the tubing loop model. Clotting was visible after a 60-min incubation of untreated islets, but not heparinized islets, in fresh nonanticoagulated human blood (Fig. 3*A*).

In the control tubing loops containing culture medium alone, only a slight drop in platelet count was observed, while nearly all of the platelets were consumed in the tubing loops containing untreated islets. The untreated islets also triggered a rise in the coagulation parameter thrombin antithrombin and an increase in C3a (Fig. 3*B–D*). In loops containing biotin (SNL)-heparinized islets, there was no macroscopic clotting, and the drop in platelet count and generation of TAT and C3a were markedly attenuated. Similar results were obtained with loops containing biotin (tissue factor P)-heparinized islets. In con-

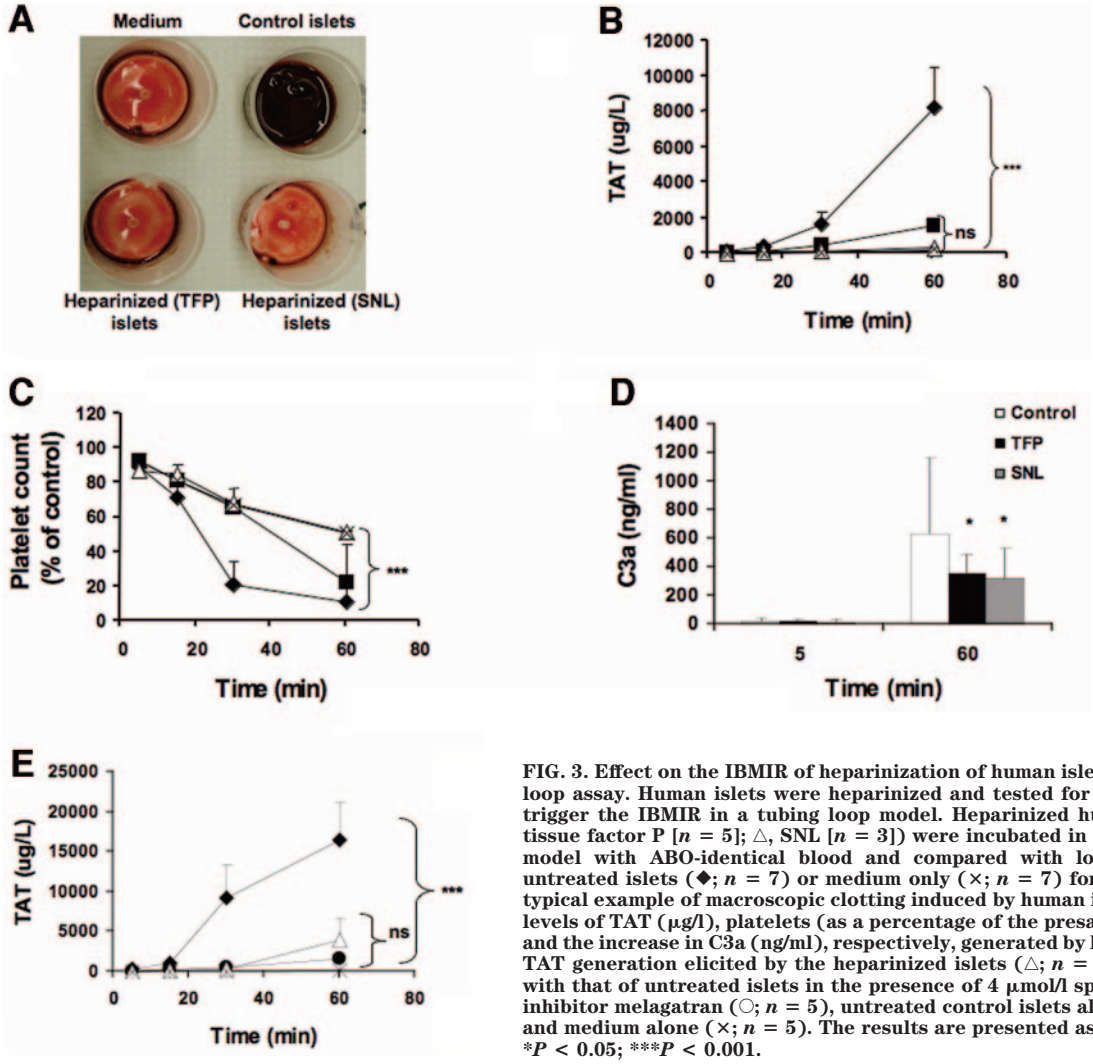

FIG. 3. Effect on the IBMIR of heparinization of human islets in the tubing loop assay. Human islets were heparinized and tested for their ability to trigger the IBMIR in a tubing loop model. Heparinized human islets (■, tissue factor P [$n = 5$]; △, SNL [$n = 3$]) were incubated in the tubing loop model with ABO-identical blood and compared with loops containing untreated islets (◆; $n = 7$) or medium only (×; $n = 7$) for ≤60 min. *A*: A typical example of macroscopic clotting induced by human islets. *B–D*: The levels of TAT (μg/l), platelets (as a percentage of the presample at 0 min), and the increase in C3a (ng/ml), respectively, generated by human islets. *E*: TAT generation elicited by the heparinized islets (△; $n = 5$) is compared with that of untreated islets in the presence of 4 μmol/l specific thrombin inhibitor melagatran (○; $n = 5$), untreated control islets alone (◆; $n = 5$), and medium alone (×; $n = 5$). The results are presented as means ± SEM. *$P < 0.05$; ***$P < 0.001$.

trol experiments, islets treated with biotin and heparin conjugate alone (excluding the avidin step) were evaluated in the in vitro loop model. The results showed no difference between untreated control islets and treated islets, indicating that heparin binding is essential for the protective effect (data not shown). The heparin coating was found to be as efficient as 4 μmol/l melagatran (Fig. 3*E*).

Intraportal transplantation of adult porcine islets. Five pairs of pigs were transplanted intraportally with heparinized or untreated adult porcine islets (Fig. 4). After 60 min, visible intraluminal macroscopic clotting was found in the portal vein branches of the pigs receiving untreated control islets ($n = 5$) (Fig. 4*A*), whereas thrombi were scarce in those receiving heparinized islets ($n = 5$) (Fig. 4*B*). We also observed a reduction in the degree of macroscopically visible infarcted areas.

In all pigs ($n = 5$) that received untreated control islets, there was a rise in TAT during the first 15-min period after infusion of the islets. In contrast, the increase in TAT was significantly attenuated in pigs that were given heparinized islets ($P < 0.05$), indicating that the IBMIR was reduced (Fig. 4*C*). Significantly increased insulin release (an indicator of cell damage) and generation of C3a were observed in a few individual animals that received untreated control islets. In the corresponding control animals that received heparinized islets, the levels of insulin and C3a was considerably lower (Fig. 4*D* and *E*).

DISCUSSION

The consistent appearance of the IBMIR in clinical islet transplantation (3,4) calls for strategies to prevent or decrease the IBMIR to minimize islet loss in the immediate posttransplantation period. We have previously shown that soluble heparin, which is regularly used during clinical islet transplantation, only marginally counteracts the IBMIR in our in vitro loop model (13). Likewise, in clinical transplantation performed with systemic heparinization, a swift increase in coagulation activation followed by a marked release of C-peptide is observed (3,4) Surface heparinization is an attractive alternative to soluble heparin. It provides a means to render the islet surface biocompatible when exposed to blood, thereby mimicking the protective characteristics conferred by heparan sulfate on the endothelial cell lining of the vascular wall. In addition to its effects on the cascade systems and the cells of the blood, the heparin coating reduces exposure of collagen and other extracellular matrix proteins on the islets that may be prothrombotic and trigger inflammation.

In the present study, we have demonstrated both in vitro and in vivo that modification of pancreatic islets with

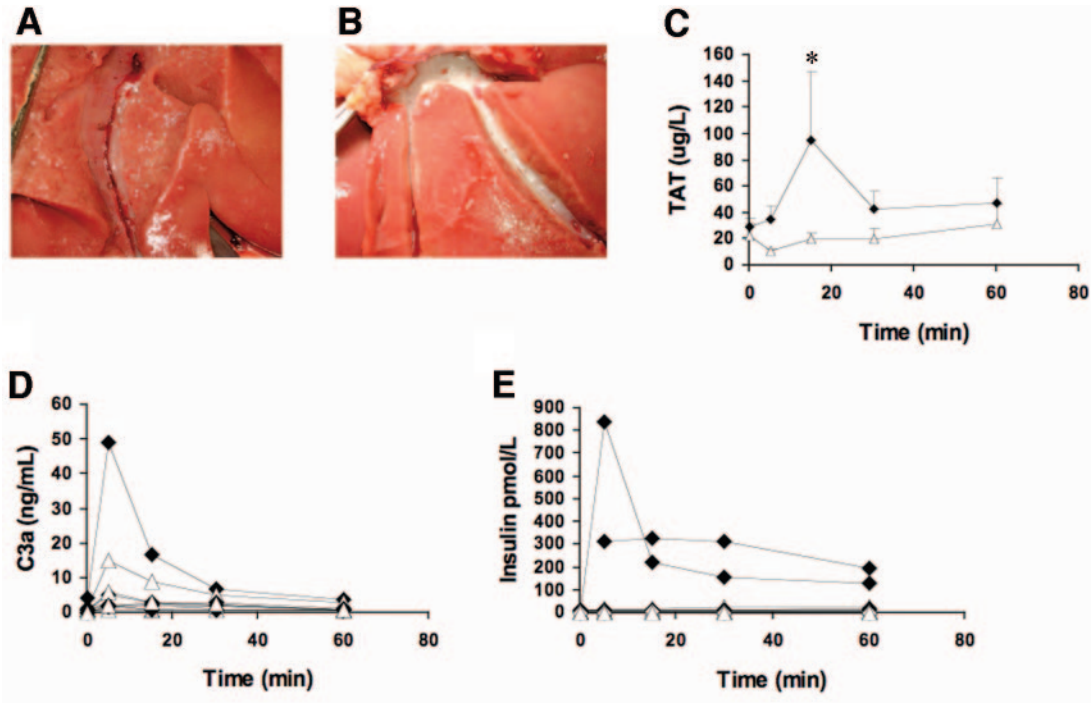

FIG. 4. Effect of heparinization of porcine islets on the IBMIR in an allogeinic islet transplantation model. Five pairs of pigs were transplanted with either heparinized or untreated islets. After 60–120 min, the experiment was stopped and the livers dissected along the portal tree. *A* and *B*: Representative pictures of a control animal that had received untreated control islets (*A*) and an animal that was transplanted with heparinized islets (*B*). *C*: Mean ± SEM ($P < 0.05$) of the TAT levels for animals given heparinized (△; $n = 5$) or untreated (◆; $n = 5$) islets. *D* and *E*: Individual levels of C3a and insulin, respectively, elicited during transplantation of heparinized (△) and untreated (◆ islets).

surface-attached heparin can reduce the deleterious IBMIR associated with islet transplantation. Using a pig model to study the immediate time period after allogeneic intraportal islet transplantation, we were able to confirm the reduced IBMIR that we had previously demonstrated in the in vitro loop model. It is reasonable to assume that improved long-term islet graft function will be obtained if the IBMIR is attenuated and more islets survive (9). To be able to show an analogous effect in immunosuppressed and diabetic large animals is, however, a major undertaking that is beyond the scope of the present study.

We have used a water-soluble macromolecular conjugate in which ~70 heparin molecules are covalently attached to a carrier carbon backbone (21) that is bound to the islet surface via biotin and avidin. Unlike previously published heparinization methods that involve multiple steps, the use of biotin and prefabricated heparin complexes reduce the handling time and the risk of damage to the islets. The present heparinization protocol is based on the fact that avidin expresses binding sites for both biotin and heparin (22), thus bolstering the multiattachment binding effect achieved by using a conjugate composed of multiple heparin molecules.

Biotin and streptavidin have previously been used to conjugate biologically active molecules to cells. In a heart transplantation model in mice, Askenasy et al. (23) successfully displayed exogenous streptavidin-conjugated proteins on the vascular endothelium of solid organ grafts and splenocytes, without any notable signs of toxicity being induced by the procedure. Similarly, no toxicity was associated with the heparin coating procedure in the present study: i.e., surface heparinization did not affect insulin release in response to a glucose challenge, and the long-term survival and function of the heparinized islets were unaffected when compared with the untreated islets transplanted into diabetic mice.

The heparinization approach is distinctly different from coatings such as microencapsulation that create a barrier for both molecules and cells. The conjugated heparin does not hinder cell migration, and therefore does not prevent revascularization, nor does it create a dead space that alters the diffusion properties and impairs the dynamics of glucose-stimulated insulin release.

Recently, our group has successfully induced a marked expression and secretion of the leech thrombin inhibitor hirudin using an adenoviral vector, indicating that human islets can successfully be modified by means of gene therapy to express molecules that can prevent the IBMIR (24). A number of proteins, such as APC (25) and CD39 (26), are also possible candidates for use. APC significantly reduced loss of functional islet mass after intraportal transplantation in diabetic mice, and islets from transgenic mice that express CD39 fail to induce coagulation in roughly one-half of the experiments when exposed to human blood. Gene therapy approaches are in many ways appealing, but they suffer from the fact that all existing procedures that introduce new DNA into the islets are associated with a risk of inducing inflammatory or even adaptive immune responses (27,28). To what extent the additional protein synthesis may affect the islets' ability to produce insulin is currently under debate and may vary depending on the transduction protocol (24,29).

Tissue factor expression is accompanied by an array of proinflammatory events that affect the islets, such as the expression of MCP-1 (6,30), interluekin-8, and macrophage migration inhibitory factor (7,31). These events are linked to ischemia after organ procurement and upregulation of

inflammatory genes occurring as a result of brain death of the donor (25,32). The heparin coating protects the affected cells from the innate immune response of the blood. Our results with successful heparin coating of artery vessel walls suggest that this heparin coating strategy may prove effective not only in reducing the adverse effects of the IBMIR in clinical islet transplantation but also in other types of cell, tissue, or organ transplantation and ischemia-reperfusion injury, without exposing the recipient to the side effects associated with systemic anticoagulant treatment (33).

In current protocols for clinical islet transplantation, a single dose of ~3,000–5,000 IU is used. Systemic administration of anticoagulants will always be associated with an increased risk of bleeding, a consideration of particular importance in islet transplantation, since the procedure often involves transhepatic puncture of a large portal vein. Despite the use of heparin, portal thrombosis is a feared complication that occurs in ~10% of transplantations (34). Covering the islet surface with heparin offers an attractive alternative with the capability of abrogating intraportal thrombosis and the IBMIR. This protection is achieved without systemic side effects, since the total amount of heparin attached to a full-size clinical islet transplant is as low as 40 IU. The technique is readily transferable to the clinical situation and holds promise for improving both the safety and outcome of clinical islet transplantation. The heparin coating may also be beneficial during the subsequent engraftment. Heparin has an affinity for a number of plasma proteins and growth factors (35), such as vascular endothelial growth factor (36) and fibroblast growth factor (37), which may facilitate revascularization and reinnervation.

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REFERENCES

1. Ryan EA, Lakey JR, Rajotte RV, Korbutt GS, Kin T, Imes S, Rabinovitch A, Elliott JF, Bigam D, Kneteman NM, Warnock GL, Larsen I, Shapiro AM: Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes* 50:710–719, 2001
2. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 343:230–238, 2000
3. Johansson H, Lukinius A, Moberg L, Lundgren T, Berne C, Foss A, Felldin M, Kallen R, Salmela K, Tibell A, Tufveson G, Ekdahl KN, Elgue G, Korsgren O, Nilsson B: Tissue factor produced by the endocrine cells of the islets of Langerhans is associated with a negative outcome of clinical islet transplantation. *Diabetes* 54:1755–1762, 2005
4. Moberg L, Johansson H, Lukinius A, Berne C, Foss A, Kallen R, Ostraat O, Salmela K, Tibell A, Tufveson G, Elgue G, Nilsson Ekdahl K, Korsgren O, Nilsson B: Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. *Lancet* 360:2039–2045, 2002
5. Moberg L, Korsgren O, Nilsson B: Neutrophilic granulocytes are the predominant cell type infiltrating pancreatic islets in contact with ABO-compatible blood. *Clin Exp Immunol* 142:125–131, 2005
6. Piemonti L, Leone BE, Nano R, Saccani A, Monti P, Maffi P, Bianchi G, Sica A, Peri G, Melzi R, Aldrighetti L, Secchi A, Di Carlo V, Allavena P, Bertuzzi F: Human pancreatic islets produce and secrete MCP-1/CCL2: relevance in human islet transplantation. *Diabetes* 51:55–65, 2002
7. Johansson U, Olsson A, Gabrielsson S, Nilsson B, Korsgren O: Inflammatory mediators expressed in human islets of Langerhans: implications for islet transplantation. *Biochem Biophys Res Commun* 308:474–479, 2003
8. Titus TT, Horton PJ, Badet L, Handa A, Chang L, Agarwal A, McShane P, Giangrande P, Gray DW: Adverse outcome of human islet-allogeneic blood interaction. *Transplantation* 75:1317–1322, 2003
9. Inverardi L, Ricordi C: Therapeutic approaches to counteract immediate blood-mediated inflammatory reaction in islet transplantation. *Transplantation* 82:312–313, 2006
10. Ozmen L, Ekdahl KN, Elgue G, Larsson R, Korsgren O, Nilsson B: Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: possible application of the thrombin inhibitor melagatran in clinical islet transplantation. *Diabetes* 51:1779–1784, 2002
11. Contreras JL, Eckstein C, Smyth CA, Bilbao G, Vilatoba M, Ringland SE, Young C, Thompson JA, Fernandez JA, Griffin JH, Eckhoff DE: Activated protein C preserves functional islet mass after intraportal transplantation: a novel link between endothelial cell activation, thrombosis, inflammation, and islet cell death. *Diabetes* 53:2804–2814, 2004
12. Johansson H, Goto M, Dufrane D, Siegbahn A, Elgue G, Gianello P, Korsgren O, Nilsson B: Low molecular weight dextran sulfate: a strong candidate drug to block IBMIR in clinical islet transplantation. *Am J Transplant* 6:305–312, 2006
13. Bennet W, Sundberg B, Groth CG, Brendel MD, Brandhorst D, Brandhorst H, Bretzel RG, Elgue G, Larsson R, Nilsson B, Korsgren O: Incompatibility between human blood and isolated islets of Langerhans: a finding with implications for clinical intraportal islet transplantation. *Diabetes* 48:1907–1914, 1999
14. Larsson R: Heparin-binding to improve biocompatibility. In *Encyclopedia of Biomaterials and Biomedical Engineering*. New York, Marcel Dekker, 2004, p. 753–761
15. Goto M, Eich TM, Felldin M, Foss A, Kallen R, Salmela K, Tibell A, Tufveson G, Fujimori K, Engkvist M, Korsgren O: Refinement of the automated method for human islet isolation and presentation of a closed system for in vitro islet culture. *Transplantation* 78:1367–1375, 2004
16. Brandhorst H, Brandhorst D, Hering BJ, Bretzel RG: Significant progress in porcine islet mass isolation utilizing liberase HI for enzymatic low-temperature pancreas digestion. *Transplantation* 68:355–361, 1999
17. Andersson A: Isolated mouse pancreatic islets in culture: effects of serum and different culture media on the insulin production of the islets. *Diabetologia* 14:397–404, 1978
18. King A, Lau J, Nordin A, Sandler S, Andersson A: The effect of capsule composition in the reversal of hyperglycemia in diabetic mice transplanted with microencapsulated allogeneic islets. *Diabetes Technol Ther* 5:653–663, 2003
19. Schmidt P, Krook H, Maeda A, Korsgren O, Benda B: A new murine model of islet xenograft rejection: graft destruction is dependent on a major histocompatibility-specific interaction between T-cells and macrophages. *Diabetes* 52:1111–1118, 2003
20. Nilsson Ekdahl K, Nilsson B, Pekna M, Nilsson UR: Generation of iC3 at the interface between blood and gas. *Scand J Immunol* 35:85–91, 1992
21. Kristensen EM, Rensmo H, Larsson R, Siegbahn H: Characterization of heparin surfaces using photoelectron spectroscopy and quartz crystal microbalance. *Biomaterials* 24:4153–4159, 2003
22. Kett WC, Osmond RI, Moe L, Skett SE, Kinnear BF, Coombe DR: Avidin is a heparin-binding protein: affinity, specificity and structural analysis. *Biochim Biophys Acta* 1620:225–234, 2003
23. Askenasy N, Yolcu ES, Wang Z, Shirwan H: Display of Fas ligand protein on cardiac vasculature as a novel means of regulating allograft rejection. *Circulation* 107:1525–1531, 2003
24. Cabric S, Elgue G, Nilsson B, Korsgren O, Schmidt P: Adenovirus-mediated expression of the anticoagulant hirudin in human islets: a tool to make the islets biocompatible to blood. *Cell Transplant* 15:759–767, 2006
25. Contreras JL, Eckstein C, Smyth CA, Sellers MT, Vilatoba M, Bilbao G, Rahemtulla FG, Young CJ, Thompson JA, Chaudry IH, Eckhoff DE: Brain death significantly reduces isolated pancreatic islet yields and functionality in vitro and in vivo after transplantation in rats. *Diabetes* 52:2935–2942, 2003
26. Dwyer KM, Mysore TB, Crikis S, Robson SC, Nandurkar H, Cowan PJ,

D'Apice AJ: The transgenic expression of human CD39 on murine islets inhibits clotting of human blood. *Transplantation* 82:428–432, 2006
27. Zhang N, Schroppel B, Chen D, Fu S, Hudkins KL, Zhang H, Murphy BM, Sung RS, Bromberg JS: Adenovirus transduction induces expression of multiple chemokines and chemokine receptors in murine beta cells and pancreatic islets. *Am J Transplant* 3:1230–1241, 2003
28. Yang Y, Li Q, Ertl HC, Wilson JM: Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J Virol* 69:2004–2015, 1995
29. He Z, Wang F, Kumagai-Braesch M, Permert J, Holgersson J: Long-term gene expression and metabolic control exerted by lentivirus-transduced pancreatic islets. *Xenotransplantation* 13:195–203, 2006
30. Beuneu C, Vosters O, Movahedi B, Remmelink M, Salmon I, Pipeleers D, Pradier O, Goldman M, Verhasselt V: Human pancreatic duct cells exert tissue factor–dependent procoagulant activity: relevance to islet transplantation. *Diabetes* 53:1407–1411, 2004
31. Waeber G, Calandra T, Roduit R, Haefliger JA, Bonny C, Thompson N, Thorens B, Temler E, Meinhardt A, Bacher M, Metz CN, Nicod P, Bucala R: Insulin secretion is regulated by the glucose-dependent production of islet beta cell macrophage migration inhibitory factor. *Proc Natl Acad Sci U S A* 94:4782–4787, 1997
32. Pratschke J, Neuhaus P, Tullius SG: What can be learned from brain-death models? *Transpl Int* 18:15–21, 2005
33. Villiger P, Ryan EA, Owen R, O'Kelly K, Oberholzer J, Al Saif F, Kin T, Wang H, Larsen I, Blitz SL, Menon V, Senior P, Bigam DL, Paty B, Kneteman NM, Lakey JR, Shapiro AM: Prevention of bleeding after islet transplantation: lessons learned from a multivariate analysis of 132 cases at a single institution. *Am J Transplant* 5:2992–2998, 2005
34. Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, Secchi A, Brendel MD, Berney T, Brennan DC, Cagliero E, Alejandro R, Ryan EA, DiMercurio B, Morel P, Polonsky KS, Reems JA, Bretzel RG, Bertuzzi F, Froud T, Kandaswamy R, Sutherland DE, Eisenbarth G, Segal M, Preiksaitis J, Korbutt GS, Barton FB, Viviano L, Seyfert-Margolis V, Bluestone J, Lakey JR: International trial of the Edmonton protocol for islet transplantation. *N Engl J Med* 355:1318–1330, 2006
35. Hirsh J, Warkentin TE, Shaughnessy SG, Anand SS, Halperin JL, Raschke R, Granger C, Ohman EM, Dalen JE. Heparin and low-molecular-weight heparin: mechanisms of action, pharmacokinetics, dosing, monitoring, efficacy, and safety. *Chest* 119:64S–94S, 2001
36. Ferrara N, Gerber HP, LeCouter J: The biology of VEGF and its receptors. *Nat Med* 9:669–676, 2003
37. Schlessinger J, Plotnikov AN, Ibrahimi OA, Eliseenkova AV, Yeh BK, Yayon A, Linhardt RJ, Mohammadi M: Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol Cell* 6:743–750, 2000